Article No. cyto.1998.0443, available online at http://www.idealibrary.com on IDEAL

# IMMUNOHISTOCHEMICAL COMPARATIVE ANALYSIS OF TRANSFORMING GROWTH FACTOR α, EPIDERMAL GROWTH FACTOR, AND EPIDERMAL GROWTH FACTOR RECEPTOR IN NORMAL, HYPERPLASTIC AND NEOPLASTIC HUMAN PROSTATES

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**Immunoreaction to TGF-α was limited to the basal epithelial cells of focal areas in the normal prostates. In benign prostatic hyperplasia (BPH) the immunostained areas were more widespread and immunolabelling was observed in both basal and columnar (secretory) cells of the epithelium. Some cells in the connective tissue stroma were also stained. In prostatic adenocarcinoma, epithelial immunostaining was even more extensive and intense than in BPH, and some stromal cells were also stained. Epidermal growth factor (EGF) immunostaining was only present in some basal cells in normal prostates. In BPH, this immunoreaction was strong in the basal cells and even stronger in the secretory cells. In prostatic cancer, the intensity of epithelial cell immunoreactivity was intermediate between that of normal prostates and that of BPH specimens. EGF-receptor immunostaining was focal and located in the basal cells in normal prostates. In BPH, labelling was also localized in basal cells but extended to wider areas. Some stromal cells appeared weakly labelled. In the prostatic carcinoma, both basal and columnar cells appeared stained and the number of immunolabelled stromal cells was higher than in BPH. The results presented suggest that, in normal conditions, EGF and TGF-α act as autocrine growth factors for the basal cells of the prostatic epithelium. In BPH this action is maintained and, in addition, the columnar cells start to secrete both factors which are bound by the basal cell receptors, giving rise to a paracrine regulation which probably overstimulates basal cell proliferation. In prostatic carcinoma, besides these regulatory mechanisms, the acquisition of EGF-receptors by the secretory cells develops an autocrine regulation which might induce their proliferation.**



Epidermal growth factor (EGF) is a 6-kDa polypeptide[1] which stimulates proliferation and keratinization of epithelial tissues in in vivo and in vitro conditions.[2] It is produced by a variety of normal cells in different tissues and has been isolated in most organic fluids including human urine,[3] prostatic secretion and seminal fluid.[4] Transforming growth factor α (TGF-α) is a 5-kDa polypeptide which shares 35% of its amino acid sequence with EGF.[5,6] Unlike EGF, TGF-α is produced by malignant cells, including those transfected by viruses.[7] The EGF-receptor (EGFR) is an intrinsic membrane glycoprotein of 170 kDa.[8] The use of the same receptor by TGF-α with a similar affinity[9] implies the intracellular transduction of the same signal.[10]

Prostatic epithelial cells synthesize EGF[11] and TGF-α.[12] Immunohistochemical localization of EGF in benign prostatic hyperplasia (BPH) and prostate cancer was reported by Fowler *et al.*[13] EGFRs have been identified in the cytoplasm of normal human prostate cells[14] and in the membrane fraction of prostatic tissue from men with BPH[15] or prostatic

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Received 29 January 1998; received in revised form 11 August 1998; accepted for publication 30 September 1998



1043-4666/99/090722+06 $30.00/0

KEY WORDS: benign prostatic hyperplasia/epidermal growth factor (EGF)/human prostate/prostatic cancer/transforming growths factor α (TGF-α)

**TABLE 1. Comparison of immunostaining intensities of the prostatic epithelium (measured as optical density) in normal prostate, benign prostatic hyperplasia, and prostatic adenocarcinoma**

| Immunostaining | Normal men | Benign prostatic hyperplasia | Prostatic adenocarcinoma |
|---|---|---|---|
| TGF-α | $5.40 \pm 1.1^a$ | $8.04 \pm 2.2^b$ | $8.30 \pm 2.4^b$ |
| EGF | $0.72 \pm 0.1^a$ | $4.49 \pm 0.9^b$ | $2.96 \pm 1.2^c$ |
| EGFR | $5.70 \pm 1.5^a$ | $6.35 \pm 2.3^a$ | $8.04 \pm 2.0^b$ |

For each immunostaining, values with different superscript letters differ significantly between them ($P \leq 0.05$).

carcinoma.[16] Comparative immunohistochemical studies on these growth factors in normal prostate, BPH and prostatic carcinoma are partial for reasons that include: the lack of normal prostatic tissue for comparison purposes, the impossibility of using an animal model, since laboratory animals do not develop prostatic carcinoma, and our relatively poor knowledge of the growth factors and their receptors that control the normal development of the prostate.

## RESULTS

The results from tests used to check the specificity of the three antibodies used were positive. No immunoreaction was observed in the negative controls incubated with pre-immune serum or anti-chloramphenicol. Staining of skin sections was positive. The results of ELISA showed a linear correlation between the increasing concentrations of the homogenized tissues and their respective optical densities. Comparison of prostates obtained during surgery with those from autopsies showed neither histological nor immunohistochemical changes. In the normal prostates, no differences in immunostaining affinity were observed between prostatic regions (central, intermediate and peripheral).

The results of the semiquantitative comparative analysis are summarized in Table 1.

In the normal prostates, immunoreaction to TGF-α was limited to focal areas that appeared intermingled with the unstained areas. In the stained areas, labelling was only observed in the basal epithelial cells (Fig. 1). In BPH, the immunostained areas were more widespread and immunolabelling was observed in both basal and columnar (secretory) cells of the epithelium. Some cells in the connective tissue stroma were also stained (Fig. 2). In prostatic adenocarcinoma immunostaining was even more extensive. Although distinction between basal cells and columnar cells were less evident than in the other groups, all the epithelial cells and some stromal cells were intensely immunostained (Fig. 3).

In normal prostates, EGF immunostaining was absent or only present in some basal cells (Fig. 4). In contrast, in BPH this immunoreaction was strong in the basal cells and even stronger in the secretory cells of the prostatic epithelium (Fig. 5). Prostatic secretions also appeared intensely labelled. In prostatic cancer sections, the EGF immunoreactivity of the epithelial cells was more intense than in normal prostates and weaker than in BPH (Fig. 6). No positive immunoeaction to EGF was observed in the stroma in any of the three prostate types studied.

In normal prostates, immunostaining to EGFR only appeared in some zones, that were intermingled with the unstained areas and was exclusively localized in the basal epithelial cells (Fig. 7). In BPH, labelling was also in basal cells but extended to wider areas. In addition, some stromal cells appeared weakly labelled (Fig. 8). In the prostatic carcinoma specimens, the immunolabelled areas were even wider and all the epithelial cells in these areas appeared intensely stained. In the connective tissue stroma, the number of immunolabelled cells was higher than in BPH and their staining was more intense (Fig. 9).

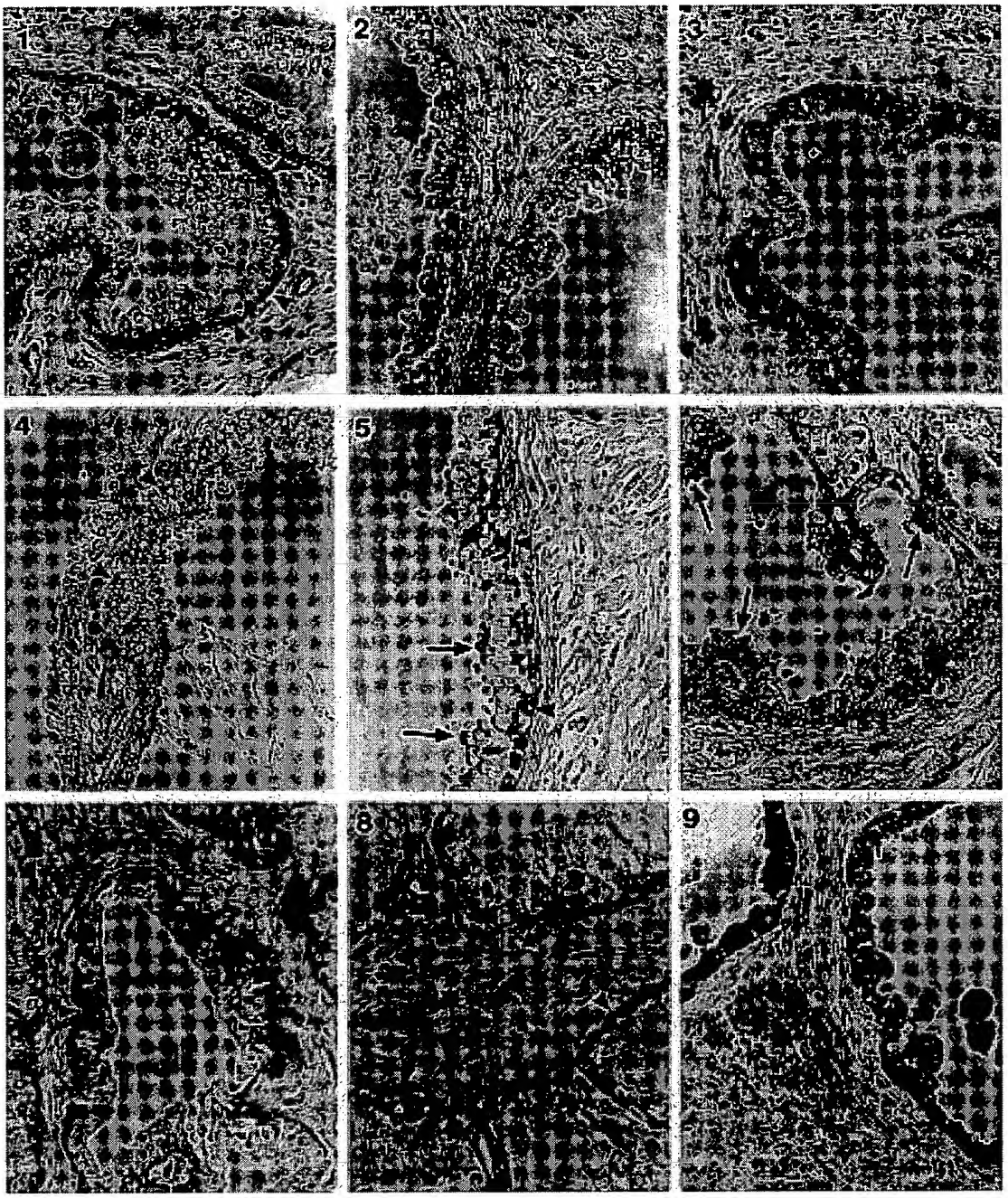
## DISCUSSION

Harper *et al.*[17] found that immunohistochemical reaction to TGF-α was very low in BPH and increased in prostate cancer. In the normal prostates evaluated in this study, TGF-α protein was expressed exclusively in the basal epithelial cells. In BPH the columnar secretory cells were also labelled. In prostatic carcinoma immunostaining was more intense than in BPH. This agrees with the opinion that autocrine production of TGF-α is characteristic of prostatic tumour cells.[18]

Previous results on EGF in BPH and prostatic cancer are controversial and in any of them comparisons with normal prostatic tissue have been performed. Using immunohistochemistry, Fowler *et al.*[13] identified EGF staining in the prostate only in 6% of BPH patients compared with 68% of prostate cancer patients. In contrast, radioimmunoassay quantitations by Habib[19] and Shaikh *et al.*[20] found significantly higher levels of EGF in patients with BPH than in prostatic cancer patients. These results agree with those reported here.

The results of previous studies on EGFR in BPH and prostatic carcinoma are also discrepant. Several authors[21–23] have reported that the number of EGFR positive cells is higher in BPH than in prostate carcinoma. In contrast, in tumour cell line cultures, EGFR expression increases with malignant potential[24,25] and

CYTOKINE, Vol. 11, No. 9 (September, 1999: 722–727)

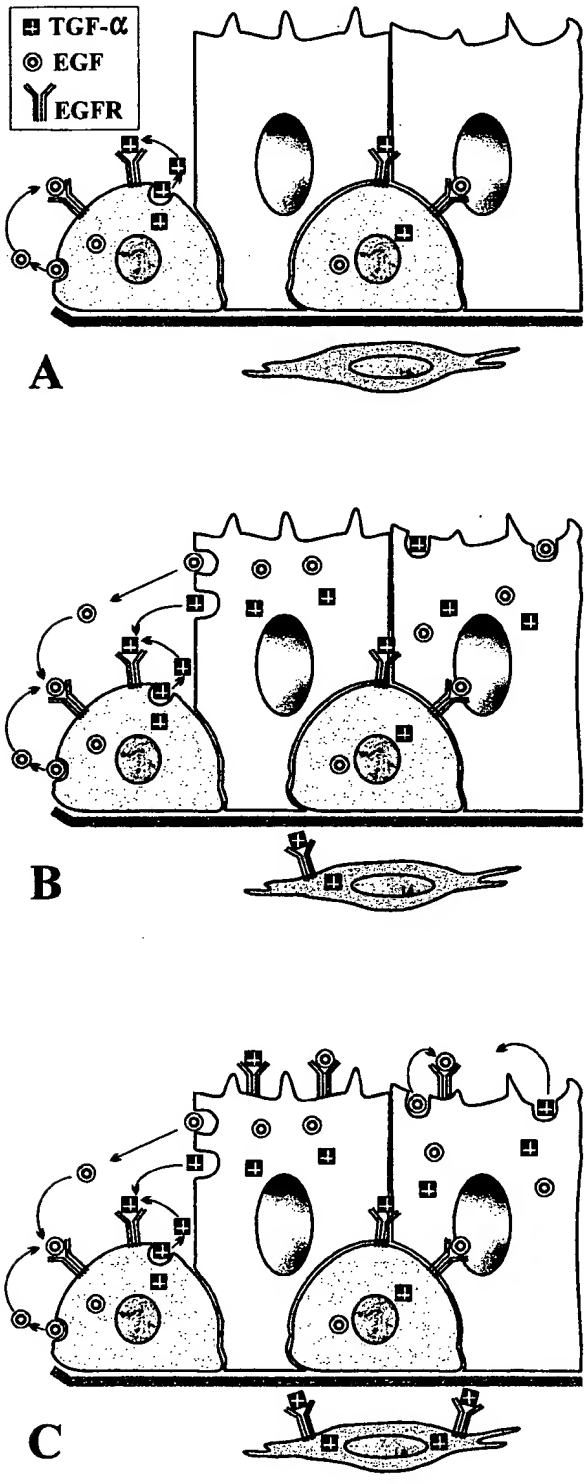



**Figures 1–9. Paraffin embedded sections of the prostate immunostained to TGF-α. (Figs 1–3), to EGF (Figs 4–6) or to EGFR (Figs 7–9).**
Figure 1: In the normal prostate the basal epithelial cells (arrowheads) are intensely immunostained. × 425.
Figure 2: Both basal cells and columnar secretory cells are stained in BPH. × 330.
Figure 3: In prostatic adenocarcinoma epithelial immunostaining is more intense and some stroma cells (star) are also stained. × 240.
Figure 4: In the normal prostate epithelial immunostaining is weak and limited to some basal (arrowheads) cells. × 320.
Figure 5: Basal cells (arrowheads) and columnar cells (arrows) are stained in BPH. × 240.
Figure 6: In prostatic adenocarcinoma epithelial cells are also immunostained (arrows) but labelling is less intense than in BPH. × 20.
Figure 7: In the normal prostate the basal epithelial cells are intensely immunostained (arrowheads). × 310.
Figure 8: In addition to basal cells (arrowheads), some stromal cells (star) are immunostained in BPH. × 500.
Figure 9: The epithelium and some stromal cells (star) are intensely immunostained in prostatic adenocarcinoma. × 300.
Reproduced at 85%.

EGFR mRNA levels were found to be slightly higher in prostatic cancer patients than in BPH.[23,26] In other studies, the levels of EGF binding were similar in both groups.[27] In the present study, the immunostaining pattern of EGF receptor differs from that of their ligands, and the intensity of EGFR immunostaining

was higher in prostatic carcinoma than in BPH and normal prostates.

There have been some studies on the intra-epithelial localization of EGFR. In an immunohistochemical study of BPH, prostatic intra-epithelial neoplasia (PIN) and prostatic carcinoma, Ibrahim *et al.*[21] and Maddy *et al.*[28] observed that EGFR immunoreactivity only appeared in the basal cells of the prostatic epithelium in both BPH and prostate cancer. In the present study, in the normal prostate and BPH, immunostaining to this receptor was only seen in basal cells whereas, in the prostatic carcinoma, the whole epithelium and some stromatic cells were strongly immunostained. Cohen *et al.*[29] found that, in normal prostates and BPH specimens, EGFR immunostaining was localized in the epithelial cells and TGF-α in the stromal cells, whereas in about half of the adenocarcinomas examined there was co-expression of the receptor and the ligand. Scher *et al.*[30] found this co-expression in epithelial tumour cells but only in specimens obtained from hormone-refractory metastases and not in primary prostatic tumours. These findings suggest that in primary tumours a paracrine pattern of growth factor stimulation predominates, whereas in androgen-independent disease a shift towards an autocrine stimulatory loop occurs.




The results reported here suggest the following hypothesis. In normal conditions, EGF and TGF-α act as autocrine growth factors for the basal cells of the prostatic epithelium (Fig. 10A). In BPH this action is maintained and, in addition, the columnar cells start to secrete both factors which are bound by the basal cells, giving rise to a paracrine regulation which probably overstimulates basal cell proliferation (Fig. 10B). In prostatic carcinoma, besides these regulatory mechanisms, the acquisition of EGFRs by the secretory cells develops an autocrine regulation which might induce their proliferation (Fig. 10C).

This interpretation is supported by previous data. It has been suggested that expression of TGF-α and its receptor by human prostate cancer cells could confer them a growth advantage, leading to its autonomous growth.[7] A marked epithelial dysplasia resembling carcinoma-in-situ cells has been observed in the anterior prostate of transgenic mice overexpressing TGF-α,[31] and suggests that overproduction of this factor contributes to continuous cell proliferation and transformation.[32] To achieve transformation, EGFR

**Figure 10. Possible actions of TGF-α, EGF and EGFR on the human prostate.**

A: In normal conditions, TGF-α and EGF are secreted by basal epithelial cells which also possess receptors for these factors (EGFR), giving rise to an autocrine stimulation of basal cell proliferation (epithelial cell renewal). B: In benign prostatic hyperplasia, the columnar secretory cells also secrete these factors, giving rise to a paracrine overstimulation of basal cells. The excessive proliferation of these cells might be the cause of hyperplasia. C: In prostatic adenocarcinoma, in addition to the autocrine and paracrine stimulations of basal cells, the columnar cells—which have developed EGFRs—undergo an autocrine stimulation and thus, proliferation of the usually non-proliferative columnar cells also might occur.

must be expressed at high levels in addition to being in the presence of activating ligand.[33] In our study, EGFR is overexpressed in all epithelial cells of the prostatic epithelium in the cancer samples. A mechanism for hyperproliferation is an unscheduled autocrine ligand synthesis by pre-malignant cells.[34] In our study, an important ligand might be TGF-α, which appears in the secretory cells of BPH specimens while it is completely absent in normal prostate and increases to higher levels in prostatic carcinoma.

## MATERIALS AND METHODS

The prostates from 30 men (aged from 60 to 80 years) were obtained by surgery. Fifteen of these men were clinically and histopathologically diagnosed of BPH, and the other 15 men presented prostatic adenocarcinoma (Gleeson grading 3). In addition, 10 prostates from 20–50-year-old men without reproductive, endocrine and related diseases were obtained between 8 and 10 h after death in autopsies. Five of these men showed BPH and the other five men presented histologically normal prostates.

Tissues were fixed for 24 h in a 0.1 M phosphate-buffered 10% formaldehyde solution, dehydrated and embedded in paraffin. Sections (5 μm in thickness) were processed following the alkaline phosphatase–conjugated streptavidin complex or the avidin–biotin–peroxidase complex methods. In brief, following deparaffinization, sections were hydrated and, in the case of the avidin–biotin–peroxidase complex method, incubated for 20 min in 0.3% $H_2O_2$ in methanol to reduce endogenous peroxidase activity. The sections were incubated overnight at 4°C with the primary antibodies (all from Santa Cruz Biotechnologies, Santa Cruz, CA, USA) diluted in Tris-buffered saline (TBS) containing 0·5% BSA. The primary antibodies used and the dilutions found to be optimal for this study were: anti-EGF antibody, at 1:25; anti TGF-α antibody, at 1:25; and anti-EGFR antibody, at 1:250. Afterwards, the sections were washed twice in TBS and then incubated with goat anti-rabbit (EGF and EGFR) or goat anti-mouse (TGF-α) biotinylated immunoglobulins (Biogenex, San Ramon, CA, USA). After 1 h of incubation at 37°C with the second antibody, the sections were incubated with phosphatase–antiphosphatase complex (Biogenex) or avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature, developed with Fast Red (Biogenex-supersensibility complex Kit) or with diaminobenzidine (Sigma, Barcelona, Spain), and mounted in Crystal/mount aqueous dry mounting medium (Biomeda, Foster City, CA, USA) or dehydrated and mounted in DePex (Probus, Badalona, Spain). Afterwards, some sections were counterstained with Mayer's haematoxylin for 2 min. Care was always taken to develop the sections of the different specimens for exactly the same time.

The specificity of the immunohistochemical procedures was checked by using negative and positive control sections. For negative control of the immunoreactions, adjacent sections of each type (normal, BPH and prostatic cancer) were incubated with pre-immune rabbit serum (for EGF and EGFR) or mouse anti-chloramphenicol (for TGF-α, a gift from P. J. S. van Kooten, Institute of Infectious Diseases and Immunology, University of Utrecht, Utrecht, The Netherlands). As positive controls, sections of skin from both mouse and human were incubated with the same antibodies. For additional control of the specificity of the antibodies, an ELISA was carried out. The protein concentration of homogenates was calculated by the Bradford method.[35] The different antigens were coated on 96 well multiplates overnight at 4°C. The plates were washed with TBS containing 0.05% Tween 20 and blocked with 1% bovine serum albumin in TBS for 1 h at room temperature, and incubated withs the different antibodies for 3 h also at room temperature. After a further wash, the peroxidase-conjugated anti-rabbit (EGF, EGFR) or anti-mouse (TGF-α) immunoglobulins were added to each well (Chemicon, Temecula, CA, USA). The interactions were visualized with 0.5% 2,2-azino-di-3-etilbenziatioazolina sulphonic acid (ABTS) (Sigma) in 100 mM citrate buffer, and was measured (optical density at 405 nm) in a spectrophotometer (Multiskan Bichromatic, Labsystems, Finland).

A histological comparative quantification of immunolabelling density in normal, hyperplastic and neoplastic prostates was performed for each of the three antibodies. Of each normal prostate, six histological sections of each region (central, intermediate and peripheral) were selected at random and the staining intensity (optical density) per unit surface area of the epithelium was measured with an automatic image analyser (MIP4 version 4.4, Consulting Image Digital, Barcelona, Spain) in five light microscopic fields using the × 40 objective. For each positively immunostained section one control section (the following in a series of consecutive sections) was also used, and the optical density of this control section was subtracted from that of the stained section. The means ± SD for the normal prostate group were calculated from the average values obtained for each prostate. The same quantitative study was carried out in the hyperplastic and neoplastic prostates, although the number of sections used was higher (23 in BPH and 29 in prostatic carcinoma) and all these sections were taken from the impaired zone. In the study, the number of sections and microscopic fields in each section necessary for calculation were determined by successive approaches to obtain the minimum number required to reach the lowest SD. The statistical significance between means was assessed by the Fisher and Behrens test.

### Acknowledgements

This work was supported by grants from the Comunidad de Madrid (AC-095), the Fondo de Investigaciones Sanitarias (98/0820) and the University of Alcalá (003/97).

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